ORIGINAL PAPER

Overexpression of a Maize Transcription Factor *ZmPHR1* Improves Shoot Inorganic Phosphate Content and Growth of *Arabidopsis* under Low-Phosphate Conditions

Xiuhong Wang · Jianrong Bai · Huiming Liu · Yi Sun · Xiangyuan Shi · Zhiqiang Ren

Abstract Maize (*Zea mays* L.) yield is limited by the poor availability of inorganic phosphate (Pi) in many arable areas worldwide. Phosphorus use efficiency (PUE) is a complex multigene trait, with a single gene contributing only a small percentage to the phenotype. Transcription factors (TFs) are very important as a single TF frequently coordinates the expression of multiple genes in response to environmental signals. Previous studies have indicated that the TFs AtPHR1 and OsPHR2 play important roles in the regulation of plant phosphorus accumulation. However, little is known about the functions of PHR-like genes in maize. In this study, a member of the MYB-CC family encoding a 449-amino acid protein, ZmPHR1, was isolated. The *ZmPHR1*::GFP fusion was localized in the nucleus, which indicates that ZmPHR1 is also a TF.

Phylogenetic tree analysis revealed that ZmPHR1 belongs to the same subfamily of MYB-CCs as OsPHR1, OsPHR2 and AtPHR1. Transgenic *Arabidopsis* lines overexpressing ZmPHR1 were used to investigate the pleiotropic effects of this gene under low Pi conditions. Overexpression of ZmPHR1 led to the upregulation of multiple genes that regulate metabolism during Pi-starvation, which in turn resulted in an elevation in Pi content in shoots. Most notably, *Arabidopsis* overexpressing ZmPHR1 showed better growth under low-Pi conditions. The results presented in this study suggest that PUE could be improved through the manipulation of the TF ZmPHR1 in maize and possibly in other species under Pi-deficient conditions.

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Introduction

Phosphate (Pi) deficiency in soil is a worldwide problem, because Pi concentrations are often within the range of 1–10 μM in 30–40 % arable land and cannot meet the demands of plant growth and development (Raghothama 1999; Schachtman et al. 1998; Tan et al. 2012). The application of phosphorus (P) fertilizer in soil is a common practice to maintain plant proper growth and high yield of crops. However, even under adequate levels of P fertilization, only 20 % or less was utilized by plants (Vance 2001). The fertilized P forms insoluble complexes with cations, particularly calcium ions in alkaline soil and aluminum or iron in acidic conditions, and is incorporated into organic matter by microbes (Vance et al. 2003). In addition, leaching and runoff of Pi from soils into water bodies result in eutrophication, which is increasingly becoming a global environmental concern (Sharply and Moyer 2000; Sims et

al. 2000). Moreover, excessive application of inorganic P fertilizers to the soil system over decades can exhaust the supply of Pi-rich minerals—a non-renewable resource—which will increase the overall cost of crop production (Vance et al. 2003; Cordell et al. 2009). In order to use genetic and breeding approaches to improve crops with complex traits such as phosphorus use efficiency (PUE), it is necessary to gain comprehensive knowledge of the regulatory mechanisms underlying Pi use, especially under Pi-deficient conditions.

Plants have evolved a variety of adaptive strategies to survive Pi deficiency. In *Arabidopsis thaliana*, transcriptome analysis indicated that approximately 29 % of the total genes are involved in Pi-starvation responses (Wu et al. 2003). Transcription factors (TFs) such as WRKY75, ZAT6, MYB62, BHLH32, AtPHR1 and PHR1-LIKE1 are the main regulators involved in regulating Pi homeostasis in various plants (Bustos et al. 2010; Chen et al. 2007; Devaiah et al. 2007a, 2007b, 2009; Rubio et al. 2001). AtPHR1, a member of the MYB-CC family, was the first transcription factor demonstrated to mediate the Pi-starvation response at the transcriptional level in *Arabidopsis*. It regulates the expression of Pi starvation-inducible (PSI) genes by binding as a dimer to the *PHR1*-specific binding sequence (P1BS) element (GNATATNC)—an imperfect palindromic sequence present in the promoter regions of these genes (Rubio et al. 2001; Franco-Zorrilla et al. 2007; Nilsson et al. 2007; Coelho et al. 2010). Pi content was increased significantly in both wild type and *phr1* mutant *Arabidopsis* plants transformed with AtPHR1, irrespective of Pi conditions (Bari et al. 2006; Nilsson et al. 2007). Recently, OsPHR2, which may function in a manner similar to AtPHR1, was isolated from rice (*Oryza sativa* L.) using the AtPHR1 protein sequence as a query. Several Pi transporters were upregulated in the shoots of OsPHR2-overexpressing plants, and correlated positively with the accumulation of Pi (Zhou et al. 2008).

Maize—one of the most economically important cultivated crops worldwide—is grown widely in both acidic and calcareous soils, where Pi fertilizers are easily absorbed by soil particles, resulting in the production of relatively insoluble and unavailable forms of Pi (Vance et al. 2003). Many maize genotypes have been screened for PUE; according to both greenhouse and field tests, only a few have turned out to be high phosphorus-utilizing types (Da Silva and Gabelman 1992; Kaeppler et al. 2000). Many important crop traits, including PUE, are complex multi-gene traits, with each gene contributing only a small proportion to the phenotype. For example, *HvPHT1-1*, which encodes a single Pi transporter, did not increase P assimilation in barley upon introduction into the genome (Rae et al. 2004). The introduction of TFs, which regulate many downstream genes, is a feasible approach to crop modification (Century et al. 2008). However, little is known about the functions of PHR-like TFs in maize compared with those in *Arabidopsis* and rice.

Here, we describe the isolation of a maize transcription factor, ZmPHR1, and its functional characterization in transgenic *Arabidopsis* plants. Overexpression of ZmPHR1 in *Arabidopsis* upregulated a series of phosphorus starvation-inducible genes and transporters, resulting in reduced sensitivity to low Pi stress, improved plant growth and Pi content in shoots in comparison with wild type. These results give hope to breeders aiming to improve PUE in crops by genetic engineering.

Materials and Methods

Pi Treatment and Growth Conditions for Maize

The previously isolated P-efficient maize inbred line 478 was used in this experiment. Seeds were surface sterilized with 75 % ethanol for 30 s, rinsed three times with sterile distilled water, and germinated in boxes filled with sterilized perlite. Seven-day-old seedlings were transferred to a continuously aerated nutrient solution with high Pi content (HP, 1 mM KH_2PO_4) for 7 days and then transferred to a low Pi solution (LP, 10 μM KH_2PO_4). The basal nutrient solution contained 2 mM $Ca(NO_3)_2$, 0.65 mM $MgSO_4$, 25 μM Fe-EDTA, 5 μM $MnSO_4$, 50 μM KCl, 2 μM $ZnSO_4$, 0.5 μM $CuSO_4$, 0.005 μM $(NH_4)_6Mo_{24}$ and 25 mM H_3BO_4. In addition, K^+ was supplied in the LP solution in the form of 0.99 mM KCl. The seedlings were grown in an artificial climatic chamber at 28 °C/18 °C (day/night) under a 16 h/8 h (light/dark) photoperiod at 100 $μmol\,m^{-2}s^{-1}$ photon flux density and approximately 70 % humidity. Roots, stems, and leaves were harvested at 0, 1, 3, 5 and 7 days after Pi starvation, frozen in liquid nitrogen, and stored at −80 °C for further analysis.

RNA Extraction and Synthesis of First-Strand cDNA

Tissue samples (50–100 mg) were ground into a fine powder in liquid nitrogen. Total RNA was then extracted from various tissues using RNAiso™ Plus (Takara, Dalian, China) according to the manufacturer's instructions. The quality and quantity of RNA were determined using a BioPhotometer (Eppendorf, Hamburg, Germany) and further confirmed by denaturing agarose gel electrophoresis. Isolated RNA was digested with DNaseI (RNase free, Takara) to remove traces of DNA. First strand cDNA was synthesized using 2.5 μg RNA in a final volume of 20 μl with a reverse transcriptase M-MLV (RNase H⁻) kit and oligo(T)$_{18}$ primer (Takara). Reverse transcription was performed at 42 °C for 60 min with a final denaturation at 70 °C for 5 min.

Cloning of ZmPHR1 by Reverse Transcription-PCR

A BLAST search was performed in the TIGR (The Institute of Genomic Research Database-TDB) Maize Database (http://maize.jcvi.org) using the amino acid sequence of AtPHR1 (NP_194590) as a query. A consensus sequence (TC346249) was obtained and used as a template to design the primer pairs (forward: 5′-AAGCAAGGTAATGAAATG-3′ and reverse: 5′-TGAATAGTGCAACCGATA-3′) with Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA). Full-length cDNA was amplified from the leaf cDNA of maize inbred line 478. RT-PCR was conducted with 2.5 ng cDNA, 2.5 U Takara Ex *Taq* polymerase (Takara), 50 μM dNTPs (Takara), 0.2 μM primers, and programmed at 94 °C for 3 min followed by 30 cycles of 94 °C for 1 min, 53 °C for 1 min, and 72 °C for 1 min. The PCR products were recovered with a DNA Fragment Quick Purification/Recovery Kit (DingGuo, Beijing, China), subcloned into a pGEM-T Easy Vector (Promega, Madison, WI) according to the manufacturer's instructions, and sequenced (Sangon, Shanghai, China).

Bioinformatic Analysis

Analysis of the *ZmPHR1* cDNA sequence was conducted with DNAMAN 6.0.40 (http://www.lynnon.com/). Multiple sequence alignment and phylogenetic analysis for *ZmPHR1* and related members of the MYB-CC subfamily were conducted with the Clustal X, MEGA 5.0 and GeneDoc programs, respectively (Tamura et al. 2011; Zhang et al. 2011). The gene structure analysis of *ZmPHR1*, *AtPHR1*, *OsPHR1* and *OsPHR2* was carried out by performing a BLAST search of the MaizeGDB genomic database (http://www.maizegdb.org) and NCBI (http://www.ncbi.nlm.nih.gov/), respectively, using the cDNA as a query.

Localization of the *ZmPHR1*::GFP Fusion Protein

To construct the *ZmPHR1*::GFP fusion protein, the coding region of *ZmPHR1* was amplified from pMD-18T Vector-*ZmPHR1* with a forward primer containing a *Sal*I site (gtcgac; 5′-gtcgac ATGAGGAAGTTTAATCTG-3′) and a reverse primer containing a *Bcl*I site (tgatca; 5′-CCG tgatca ACTATCTTGCAGTTT-3′). *Bcl*I and *Bam*HI are isocaudomers. The PCR products were inserted between the *Sal*I and *Bam*HI sites in the pJIT163-GFP vector (pJIT163 vector, http://www.pgreen.ac.uk) with two CaMV 35S promoters (Fig. 3a). The pJIT163-*ZmPHR1*::GFP and pJIT163-GFP vectors were transferred into onion epidermal cells using a Biolistic PDS–1000/He particle delivery system (Bio-Rad, Hercules, CA), The GFP signal was detected using a laser confocal scanning microscope (LCSM; Olympus, Tokyo, Japan) at a wavelength of 488 nm.

Expression Analysis of *ZmPHR1* by Semi-quantitative RT-PCR and Quantitative PCR

Semi-quantitative RT-PCR and quantitative PCR (qPCR) were conducted to analyze the expression of the *ZmPHR1* gene in roots, stems and leaves of maize seedlings at various time points (0, 1, 3, 5 and 7 days after LP-treatment). Maize α-tubulin was used as a reference gene to normalize both cDNA concentrations (RT-PCR) and expression data (qPCR). The primers for amplifying α-tubulin were 5′-ACTTCATGCTTTCGTCCTACGCTCCA-3′ (forward) and 5′-CTGGGAGGCTGGTAGTTGATTC-3′ (reverse). *ZmPHR1* gene-specific primers A (PA, designed for amplifying complete cDNA sequence) were 5′-AAGCAAGGTAATGAAATG-3′ (forward) and 5′-TGAATAGTGCAACCGATA-3′ (reverse). *ZmPHR1* gene-specific primers B (PB, designed for qPCR) were 5′-CACCCTTTATTTCTCAGTCATCCAA-3′ (forward) and 5′-TCATTTTGTGTAGCACTCTCATCAG-3′ (reverse). Semi-quantitative RT-PCR was conducted with 2.5 ng cDNA, 2.5 U Takara Ex *Taq* polymerase (Takara), 50 μM dNTPs (Takara) and 0.2 μM primers (PA). The PCR program was 94 °C for 3 min followed by 30 cycles of 94 °C for 1 min, 58–60 °C for 1 min, and 72 °C for 1 min. The PCR products were separated on 1 % agarose gels. Next, qPCR was performed on a Bio-Rad platform (CFX96) using an SYBR Green detection chemistry kit (SYBR® Premix Ex *Taq*™, Takara). The qPCR was performed in a final volume of 20 μL in a mixture containing 10 μL 2× SYBR Premix Ex *Taq*™, 300 nmol each of specific forward and reverse primers (PB) and 40 ng cDNA. The amplification program was 95 °C for 5 min followed by 40 cycles of 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 1 min. After each PCR reaction, the specificity of the amplification was controlled by melting curve analysis (from 65 to 95 °C). The relative expression levels of the target genes were measured using the $2^{-\triangle\triangle Ct}$ method of relative quantification ($\triangle\triangle Ct=[Ct\ gene\ -\ Ct\ tubulin]$) (Livak and Schmittgen 2001).

Transformation of Arabidopsis and Identification of Transgenic Plants

The fragment containing the 2×35S CaMV promoter, *ZmPHR1*, GFP, and terminator in the vector pJIT163-*ZmPHR1*::GFP was digested with *Kpn*I and *Xho*I and inserted into the binary vector pCAMBIA1300 between the *Kpn*I and *Sal*I sites (*Sal*I and *Xho*I are isocaudomers; Fig. 3a). The constructed binary vector, p*ZmPHR1*::GFP, was transferred into *Agrobacterium tumefactions* strain GV3101 using the freeze-thaw method.

Next, p*ZmPHR1*::GFP was introduced into *Arabidopsis* using the floral dip method (Clough and Bent 1998).

Arabidopsis thaliana (Ecotype Columbia) was planted in peat mould and vermiculite at a ratio of 3:1, grown at 23 °C, 65 % humidity, under a 16 h light/8 h dark photoperiod at 100 μmolm^{-2}s^{-1} photon flux density. The seeds harvested from the transformed plants were surface sterilized with 75 % ethanol for 30 s, followed by 20 % hypochlorite for 20 min. The seeds were germinated and grown in Murashige and Skoog medium containing 0.8 % agar, 1 % sucrose and 50 mgL^{-1} hygromycin B (Sigma) to select transgenic seedlings. DNA and RNA from the shoots of hygromycin-resistant plants were extracted for PCR, RT-PCR and qPCR with *ZmPHR1*-specific primers to check for target gene insertion and expression. Homozygous and overexpressing *ZmPHR1* transgenic lines were identified from T$_3$ plant lines.

Transgenic and wild-type *Arabidopsis* plants were used to detect *ZmPHR1* green fluorescence protein (GFP) under a living body image-forming system (NightOWL IILB 983, Bad Wildbad, Germany). GFP expressed in root apical meristems was detected through a laser scanning confocal microscope (LSCM) at a wavelength of 488 nm.

Treatment of *ZmPHR1* Transgenic and Wild-type Arabidopsis Plants

ZmPHR1 transgenic and wild-type *Arabidopsis* seeds were planted on sponges moistened with sterile water. Two weeks after germination, the plants were transferred into pots, each containing ten plants and 2.0 L aerated HP nutrient solution (1 mM Pi). The basal nutrient solution composition was the same as that used for maize culture. Each treatment was performed in triplicate. After 2 weeks of HP culture, half of the seedlings were transferred to LP nutrient solution (10 μM Pi) and the other half were maintained in HP

nutrient solution. After 7 days of P-starvation treatment, shoots and roots from plants of both genotypes were harvested for qPCR. Two weeks later, five plants from each replicate were dried in an oven prior to biomass weighing and total P measurement, and the other five plants were instantly frozen in liquid nitrogen for inorganic phosphate (Pi) measurement (Nilsson et al. 2007).

Measurement of Total P and Pi

Inorganic Pi contents were measured following previously described methods (Nanamori et al. 2004). Frozen samples were ground using an ice-cold mortar and pestle and placed into a 2.0 mL centrifuge tube. The ground powder was then incubated in 2 % (v/v) glacial acetic acid at 42 °C for 30 min. After centrifugation at 12,000 rpm for 2 min, the Pi content of the supernatant was measured using the Mo (ammonium molybdate)-Sb (antimony potassium tartrate)-Vc (ascorbic acid) method; 0.4 % (w/v) ammonium molybdate melted in 0.5 M H$_2$SO$_4$ was mixed with 0.05 % antimony potassium tartrate (solution A), and 10 % ascorbic acid (solution B; A:B= 6:1) was then added to the mixture. One milliliter of this working solution was added to 1 mL of the sample solution, and the mixture was incubated in a water bath at 37 °C for 1 h. After cooling for 5 min at 4 °C, the absorbance was measured at 820 nm. The Pi concentration was calculated by normalization of fresh weight. The total plant P content was analyzed by the Mo (ammonium molybdate)-Sb (antimony potassium tartrate)-Vc (ascorbic acid) method after the plant material was digested with H$_2$SO$_4$-H$_2$O$_2$ at 300 °C, and normalized by dry weight (Zhou et al. 2008).

The results of above measurements were subjected to analysis of variance (ANOVA) to determine the significance of the differences in responses to treatment between genotypes. Least significance difference (LSD) post-hoc tests

Table 1 Various phosphorous starvation-response genes in *Arabidopsis* and their gene-specific primers

Gene	Accession number	Forward primer	Reverse primer
AtActin	At5g09810	5′-TAACAGGGAGAAGATGACTCAGATCA-3′	5′-AAGATCAAGACGAAGGATAGCATGAG-3′
AtIPS1	AT3G09922	5′-CTGATTCAGACTGCGAGTTTTG-3′	5′-GGAGTGGGTACAACCCAAACA-3′
AtPS2	AT1G73010	5′-CACGACTTCACTAAATCCCCTC-3′	5′-GCTCCATCTCCTAGATAGATCATTTT-3′
AtPS3	AT3G47420	5′-CTCTACTGGCTCCCATTTTACG-3′	5′-CTCTTGCACCAATTCGGTCT-3′
AtRNS1	AT2g02990	5′-TGATGCCTCTAAACCATTCGAT-3′	5′-TACCATGCTTCTCCCATTCG-3′
Pht1;2	AT5g43370	5′-GACTACCCACTTTCTGCCACCAT-3′	5′-CTTTCCTCAAGCTCGATATCTGT-3′
Pht1;4	AT2g38940	5′-ACCCAATGCTACAACCTTCG-3′	5′-CTGGGTTCTGAGCCAAGTAC-3′
Pht1;8	AT1g20860	5′-GCTGCATTGACGTTCTATTGG-3′	5′-CGATGATGGCTCCTAATTCC-3′
Pht1;9	AT1g76430	5′-GTTCAAGAACGCTGGAGAGG-3′	5′-TAGGAACCAATTGGCTGAGG-3′

were conducted to determine the differences between the individual treatments (DPS for Windows; Hangzhou RuiFeng Information Technology, Hangzhou, Zhejiang, China; Tang and Zhang 2012).

Fig. 1 The full-length cDNA (1,703 bp) and deduced amino acid sequence (ORF from 67 to 1,416 bp) of ZmPHR1. The MYB domain is indicated in *bold* letters, and a predicted coiled-coil (CC) domain is shown in *bold-italics*. Nuclear localization signal is underlined

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1    AAGCAAGGTAATGAAATGTACCAGAAGTGACTTATGGAAAGGAAGGAGCTTAGTTCACTG
61   TTCTGAATGAGGAAGTTTAATCTGATGCAGTCTCAAAAGAGCAGAGTTTTGGGAGCAATG
21                   M R K F N L M Q S Q K S R V L G A M
121  TCATCCTCTTTGCCTATTCTGCCAAATCCTTTGAAAGGGAGCTTCCCAAGGCCTCATAAC
41       S S S L P I L P N P L K G S F P R P H N
181  CCCCAGCATATTCCTATGTTGAGGCAGCTGCCTGATGACTCTATGCCCTTGTGTATTGAC
61       P Q H I P M L R Q L P D D S M P L C I D
241  ACACATCAGTCTGTTAGTTTGCACCCAAGAGCTGGTGTTATCGGGGTACCATATTCAGGC
81       T H Q S V S L H P R A G V I G V P Y S G
301  TACACTGCTAGTCCACTTGATTCTGTGTCTAACCTTGATAGCCAGACAATGGCTGCACCC
101      Y T A S P L D S V S N L D S Q T M A A P
361  TTTATTTCTCAGTCATCCAATTTTGAAGCTCTCCAGTCTCTATCTGATAATACCCCAGAA
121      F I S Q S S N F E A L Q S L S D N T P E
421  ACACACACTAAGGCAGCCTGGTTCACATCTTCTATGGATGTTTCACCACTCAACACAGAT
141      T H T K A A W F T S S M D V S P L N T D
481  AATATTGCTGCTTCTGATGTTAATCAAATCCATAGTATACGTCCTGCTATGACATCTGAT
161      N I A A S D V N Q I H S I R P A M T S D
541  GAGAGTGCTACACAAAATGATTGGTGGGCAGATATAATGAATGATGATTGGAAAGATATT
181      E S A T Q N D W W A D I M N D D W K D I
601  CTTGACGCAACAGCTACTGATTCCCACTCAAAAGCCATGATTCAAATTTCCAACTCAGCT
201      L D A T A T D S H S K A M I Q I S N S A
661  ACATCACTACCTGCAGTAAATCAGTCAGCTTCATCTCATAGTAGGGAGATTTGTCCTGTT
221      T S L P A V N Q S A S S H S R E I C P V
721  GCTAGTCCTCCCAATAGCAGCAATGCTTCAGTTGCCAAACAACGGATGAGATGGACCCCA
241      A S P P N S S N A S V A **K Q R ■ R T T P**
781  GAACTCCATGAATGTTTTGTAGATGCTGTAAATCAGCTTGGCGGTAGCGAAAAAGCTACT
261      **E L H E C F V D A V ■ Q L G G S E K A T**
841  CCTAAGGGTGTGCTAAAGCTTATGAAAGTTGATGGTTTGACTATATATCATGTCAAAAGC
281      **P K G V L K L ■ K V D G L T I Y H V K S**
901  CACCTGCAGAAGTACCGCACAGCCCGCTATAAACCAGACCTGTCAGAAGGTACATCGGAA
301      **H L Q K Y R T A R Y K P D L S E G T S E**
961  AAAAGGACAGCCACTGAAGAGCTGGTCCTGGACCTGAAAACGAGCATGGATCTTACTGAA
321      K R T A T E E L V L D L K T S M D L T *F*
1021 GCGCTGCGCCTTCAGATGGAAGTCCAGAAACGGCTTCATGAACAGCTTGAGATTCAGAGA
341      *A L R L Q M E V Q K R L H E Q L E I Q R*
1081 AAATTGCAGTTGCGGATTGAAGAGCAAGGAAAGTATCTGCAGATGATGTTTGAAAAGCAG
361      *K L Q L R I E E Q G K Y L Q M M F E K* Q
1141 AGTCAATCGAGCACGGAGAAAGTCCAGGATCCATCCTCAAGGGATACAACAGCTAAACCG
381      S Q S S T E K V Q D P S S R D T T A K P
1201 TCATCTAATCAGAGCCAGTCTACAAACAAGGATAGTGGTGCAACCATGGACCCAAATGGA
401      S S N Q S Q S T N K D S G A T M D P N G
1261 ACGGGAGACATCGCACGGACTGCAGAACTGGGAGAACGTTCGTCTGAACTAGGTGTAAAA
421      T G D I A R T A E L G E R S S E L G V K
1321 CAGAAACTTGTAGAGATCGAATCTGGTACCGAAGTAGCCACAGGCGACAGATCTAAGATC
441      Q K L V E I E S G T E V A T G D R S K I
1381 TCCCAAGAGAAGCGGCGCAAACTGCAAGATAGTTAACATATCGGTTGCACTATTCAGACA
461      S Q E K R R K L Q D S *
1441 TAGCTGTTCAAACCGTTTGCTTGGTGAAAAATTGGTAATCTGTGTGTCGAATTCGACTAT
1501 TGATCCGTAAGTCCATTGGTGAGTTGTACCGCTCTTCAGTTAGCCATATATATTTATAAA
1561 GGATCCAAAAGAGGACCAAAGGAGAACTTCCTGGCAGCCGTGTTTATAACATTAGCAATA
1621 AGAGCTATCCCCGAAGTTATGGGGTAAAATAAGGTCTTGAAATTTAGCTTTCTTTCTGAA
1681 TTCCTCCTTTTCTTTTGTTTGGG
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Expression Analysis of Phosphorous Starvation Response Genes by qPCR

Shoots and roots of the transgenic T₃ and wild type plants after low Pi (LP) treatment for 7 days were used for qPCR. The expression of *AtIPS1*, *AtPS2*, *AtPS3*, *AtRNS1*, *Pht1;2*, *Pht1;4*, *Pht1;8* and *Pht1;9* were analyzed. The specific primers for different genes are listed in Table 1. The above genes were amplified and the relative expression levels of the target genes were measured as described before.

Results

Cloning and Sequence Analysis of *ZmPHR1*

To isolate the full-length cDNA encoding maize PHR1, the *AtPHR1* (NP_194590) amino acid sequence was used as a query to BLAST the TIGR Maize Database (http://maize.jcvi.org). A target fragment 1,703 bp long was obtained. Sequence analysis revealed that it included an open reading frame (ORF) of 1,347 bp, which encoded a predicted protein of 449 amino acids with a calculated molecular weight of 49.59 kDa and theoretical pI value of 6.20 (Fig. 1). Multiple sequence alignment results showed that the predicted amino

acids sequence shared 43 %, 64 %, and 45 % identity with the amino acid sequences of AtPHR1, OsPHR1 (AK063486), and OsPHR2 (AK100065), respectively. The results also indicated that the four amino acid sequences were highly conserved in both MYB DNA-binding domain (BD) and CC domain (Fig. 2a). The newly identified gene was designated *ZmPHR1* and deposited with GenBank under the accession number JF831533.

Phylogenetic analysis was performed based on the neighbor-joining method and bootstrap analysis (n=1,000) using 19 protein sequences, including the sequences of the four genes described above, CrPSR1 in *Chlamydomonas reinhardtii* (Wykoff et al. 1999) and another 14 proteins with a conserved MYB and CC Domains in *Arabidopsis* (Fig. 2b). They were separated into two main groups. ZmPHR1 was clustered in the same group as AtPHR1, OsPHR1 and OsPHR2. ZmPHR1 was sub-grouped with OsPHR1 and OsPHR2, which was consistent with their close evolutionary relationship (Fig. 2b).

The *ZmPHR1* gene sequence was obtained by BLAST against the MaizeGDB database. *ZmPHR1*, which was 3,776 bp in length and contained seven exons and six introns, was located on maize chromosome 1. The gene structure of *ZmPHR1* had a similar splicing pattern as *AtPHR1*, *OsPHR1* and *OsPHR2* (Fig. 2c).

Fig. 2a–c MYB-CC domain, phylogenetic and structural analysis of *ZmPHR1*. a Alignment of MYB-CC domain of *ZmPHR1* with *AtPHR1*, *OsPHR1* and *OsPHR2*. b Phylogenetic tree analysis of *ZmPHR1* with other related MYB-CC proteins from *Arabidopsis*, *Oryza sativa* and *Chlamydomonas reinhardtii*. A phylogram of proteins sharing the MYB and predicted CC domains was constructed using the Clustal X program and the neighbor-joining method (Zhang et al. 2011). *Bar* 0.2 substitutions per site. c Diagram of *ZmPHR1*, *OsPHR1*, *OsPHR2* and *AtPHR1*. Exons and introns are indicated as *black* and *white boxes*, respectively. Numbers indicate the length of each exon and intron. Start codon (ATG) and stop locations are shown by *arrowheads*
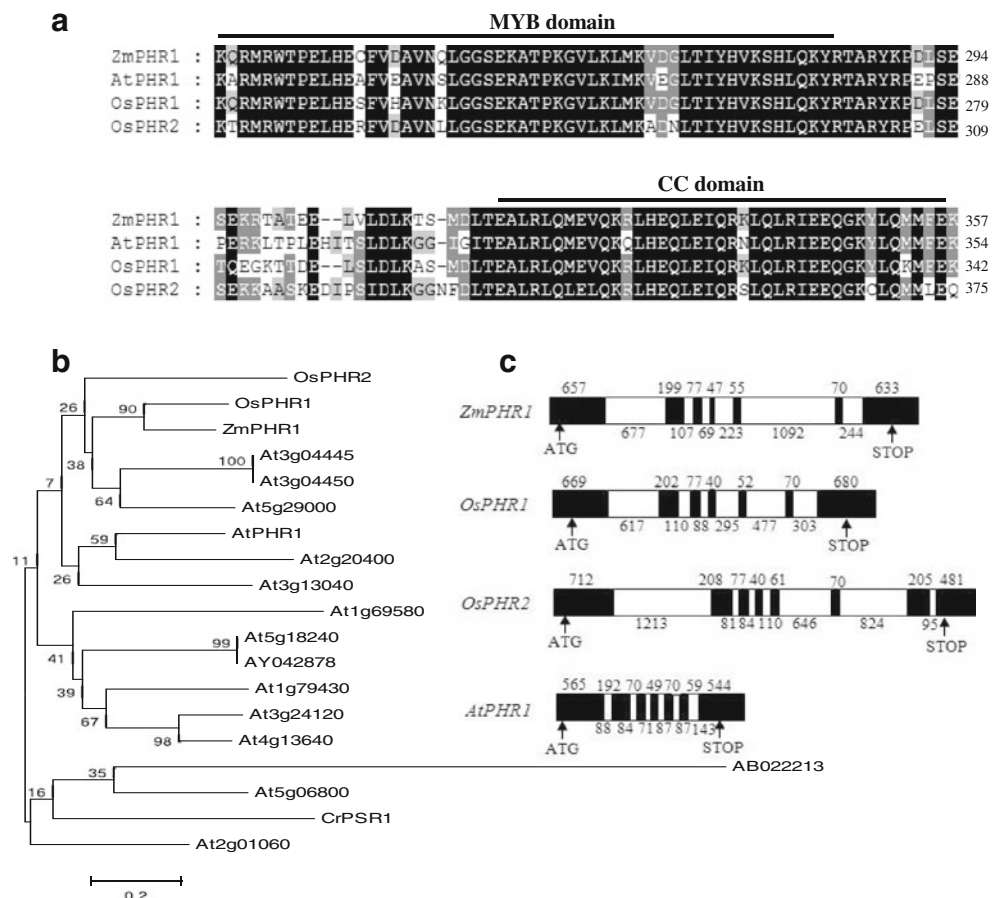
Fig. 3a,b Subcellular localization of *ZmPHR1* in onion epidermal cells. **a** Diagram of the fusion protein expression vector (JIT163-ZmPHR1::GFP), which contains an in-frame fusion protein with the ORF of *ZmPHR1* and GFP under the control of two CaMV35S promoters (35S-P). *CaMV-T* CaMV Terminator. **b** Subcellular localization of ZmPHR1::GFP fusion protein and GFP protein (control) in the cell. The green fluorescence of *ZmPHR1*::GFP was detected in the nucleus (*a*, *b* and *c*) and that of GFP was detected in the cytomembrane and nucleus (*d*, *e* and *f*). The epidermal cells selected for the confocal images (*b* and *e*) are the same as the images (*a* and *d*) taken with transmitted light. Images *c* and *f* are images merged by *a* and *b*, *d* and *e*, respectively. *Bar* 100 μm
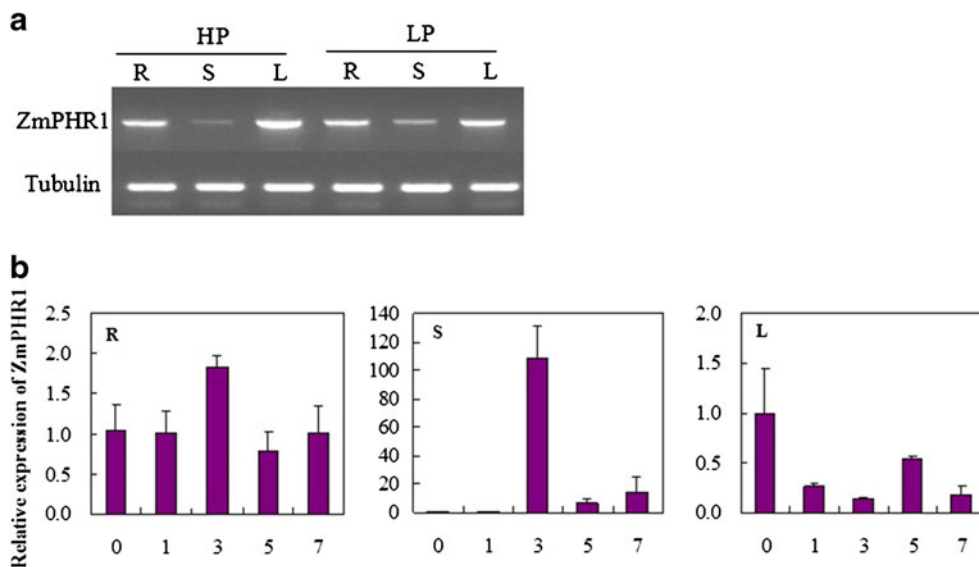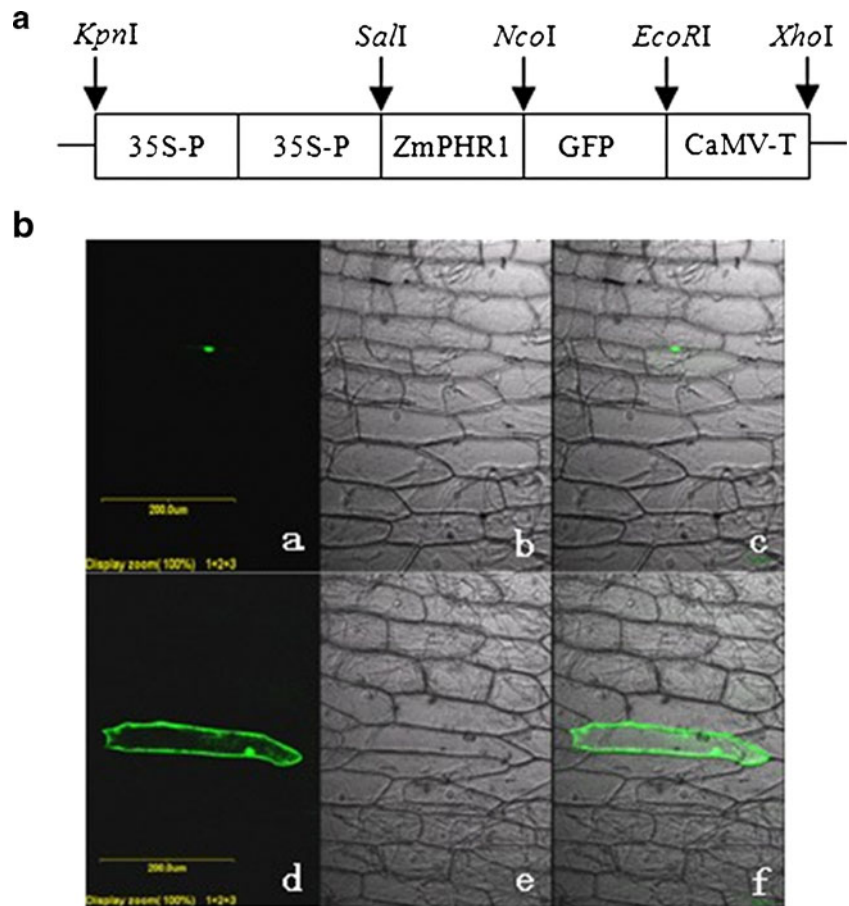

Fig. 4 a RT-PCR analysis of *ZmPHR1* gene expression. Leaves exhibited the highest levels of *ZmPHR1* transcripts among the three tissues. Lanes: *R* Roots, *S* stems, *L* leaves. Maize seedlings were grown hydroponically in high Pi medium (HP, 1 mM Pi) for 7 days and then transferred to medium with high Pi or low Pi (LP, 10 μM Pi), where they were grown for an additional 7 days. Expression of *ZmPHR1* was detected by RT-PCR analysis. *Tubulin* was used as a reference gene. **b** qPCR analysis of *ZmPHR1* gene expression in plants. Seven-day-old seedlings in HP nutrient medium were transferred LP medium, and expression of *ZmPHR1* was detected in roots (*R*), stems (*S*), and leaves (*L*) at 0, 1, 3, 5 and 7 days after Pi starvation. The expression of *ZmPHR1* expression at 0 day was used to calibrate the samples. *Error bars* Standard deviation (*n*=3)
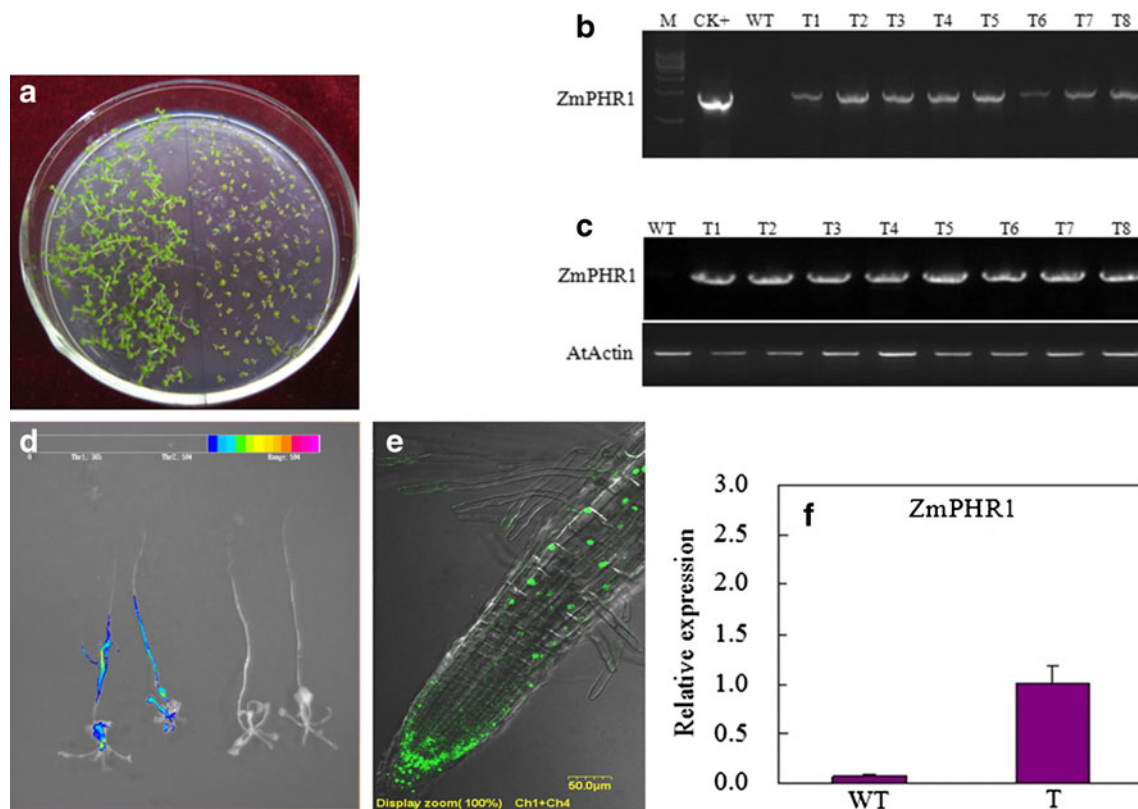
Fig. 5a–f Expression of *ZmPHR1* in transgenic *Arabidopsis*. **a** T$_3$ progeny plants of transgenic *Arabidopsis* with hygromycin-resistant (*left*), and wild-type *Arabidopsis* (*right*). **b** Verification of transgenic integration by PCR. Lanes: *M* 1 kb DNA ladder, *CK* positive control (recombinant plasmid), *WT* wild type plant, *T1–T8* transformed plant lines. **c** Expression of transcripts in transgenic plant lines (*upper panel*) and *AtActin* (reference gene; *lower panel*) evidenced by RT-PCR. **d** Green fluorescence was detected in the transgenic plants (*left*), but not

in control plants (*right*), using a living body image-forming system (Night OWL II LB 983), indicating the expression of *ZmPHR1* protein in transgenic plants. **e** Green fluorescence was detected in the nucleus of a transgenic root tip with a confocal microscope. *Bar* 50.0 μm. **f** Relative quantitative expression of *ZmPHR1* in wild-type (*WT*) and transgenic lines (*T*) under HP conditions. *AtActin* was used as a reference gene. Transgenic line was used for calibration. *Error bars* Standard deviation (*n*=3)

ZmPHR1 is a TF

In order to determine whether *ZmPHR1* encodes a transcription factor like AtPHR1 and OsPHR2, the *ZmPHR1*∷GFP fusion protein construct (JIT163-*ZmPHR1*∷GFP) was generated by fusing the *ZmPHR1* coding sequence to the 5′ end of the GFP gene (Fig. 3a). This construct was then introduced into onion epidermal cells by particle bombardment. The fluorescence signal was located predominately in the nuclei of *ZmPHR1*∷GFP transformed cells (Fig. 3b: *a, b, c*). In contrast, the GFP fluorescence signal was distributed throughout entire control cells that were transformed with the GFP vector (Fig. 3b: *d, e, f*). The results demonstrated that ZmPHR1 was a nuclear protein.

Expression Patterns of *ZmPHR1*

To investigate the spatial expression patterns of *ZmPHR1* in maize under different Pi conditions, semi-quantitative RT-PCR analysis was performed on different seedling tissues 7 days after Pi treatment. *ZmPHR1* showed a constitutive

expression pattern in all three tissues, with high expression in leaves and roots and weak expression in stems of plants subjected to either HP or LP treatment (Fig. 4a).

The temporal expression patterns of *ZmPHR1* in plants under Pi-starvation stress were further studied by qPCR. Expression levels of *ZmPHR1* were quantified in various tissues at different timepoints (0, 1, 3, 5 and 7 days) during
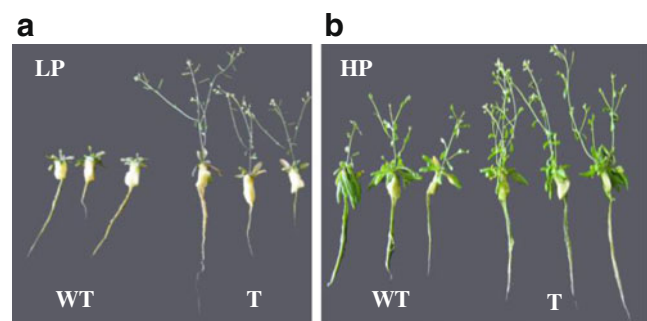

Fig. 6 Phenotypes of transgenic *Arabidopsis* overexpressing *ZmPHR*1 (*T*) and wild-type lines (*WT*) grown hydroponically in **a** low Pi (LP, 10 μM) conditions and **b** high Pi (HP,1 mM) conditions

Pi-starvation treatment. In roots and stems, the expression level of *ZmPHR1* increased by 1.84-fold and 108.86-fold 3 days after Pi starvation, respectively, followed by a decrease at 5 days. By contrast, *ZmPHR1* expression in leaves was reduced dramatically at the beginning of LP-treatment, followed by an increase at 5 days to approximately half of the level observed at 0 day (Fig. 4b).

Transformation of Arabidopsis and Identification of *ZmPHR1*-Overexpressing Transgenic Plants

Arabidopsis lines overexpressing *ZmPHR1* were generated to identify the function of *ZmPHR1* in vivo. A binary vector containing the 2×35S::*ZmPHR1*::GFP fusion protein was constructed and introduced into *Arabidopsis*. Hygromycin-resistant seedlings were selected, and T_3 progeny were generated (Fig. 5a). T_3 progeny plants from eight independent transgenic lines (T1–T8) were confirmed by PCR (Fig. 5b) and RT-PCR (Fig. 5c). Using a living body image-forming system, green fluorescence was detected, as expected, only in the transgenic *Arabidopsis* plants (Fig. 5d). Moreover, green fluorescence was detected in the root tips of transgenic plants under an LSCM, which further proved that ZmPHR1 was located in the nucleus and distributed mainly in root apical meristems (Fig. 5e). A 13.06-fold higher *ZmPHR1* transcript level was identified by qPCR (Fig. 5f) in T1 plant lines than in their WT counterparts under HP conditions.

Overexpression of *ZmPHR1* Improves growth and Pi Content in Shoots under LP Conditions

The phenotype of *ZmPHR1*-overexpressing lines and WT under different Pi levels was investigated to characterize the function of *ZmPHR1*. Vegetative growth of both genotypes was inhibited under LP conditions compared to HP conditions (Fig. 6). The shoot sizes and dry matter yield of *ZmPHR1*-overexpressing lines were much greater (Fig. 6a) and higher ($P<0.05$, Table 2), respectively, than those of WT, especially under LP conditions. Although there was no significant difference in root dry matter yield between the two genotypes under either HP or LP conditions, the root-to-shoot ratio, an
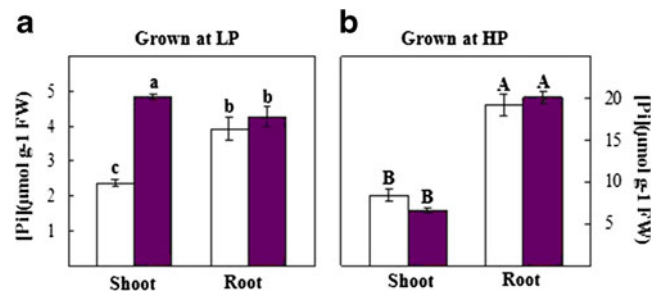

Fig. 7a,b The distribution of free phosphate (Pi) in shoots and roots of wild-type (*white columns*) and ZmPHR1-overexpressing (*purple columns*) *Arabidopsis* plants. **a** Grown in hydroponics at LP (10 μM Pi) conditions, the level of Pi accumulation in the shoots of *ZmPHR1*-overexpressing plants was almost twice that in WT with significant difference. No significant difference in Pi accumulation was observed in the roots between both genotypes. **b** Grown hydroponically at HP (1mM Pi) conditions, no significant difference in Pi accumulation was observed in the shoots and roots between both genotypes. Letters above the SE values of a particular parameter indicate whether the genotype had a significant influence within each P level ($P < 0.05$), as determined by analysis of variance (one-way ANOVA) followed by least significance difference (LSD) post-hoc tests. *FW* Fresh weight. *Error bars* Standard error ($n = 3$)

important indication of plants in response to Pi starvation, of *ZmPHR1*-overexpressing lines was significantly lower than that of WT under LP conditions ($P<0.05$, Table 2). This indicates that the transgenic plants were less sensitive to LP than WT plants, which led to better growth of transgenic vs WT plants.

Total P content of shoots and the distribution of Pi in roots and shoots were also analyzed in hydroponically grown *ZmPHR1*-overexpressing and wild-type plants (Table 2, Fig. 7). Total P contents of shoots were slightly higher in the *ZmPHR1*-overexpressing plants than in the WT plants, albeit with no significant difference, irrespective of Pi conditions. However, the level of Pi accumulation in the shoots of *ZmPHR1*-overexpressing plants was almost twice that in WT under LP conditions with significant difference ($P<0.05$; Fig. 7a). No significant difference in Pi accumulation was observed in the roots between both genotypes (Fig. 7a, b). These results suggest that overexpression of *ZmPHR1* increased Pi content in shoots under LP conditions.

Table 2 Shoot and root dry weight, shoot-to-root ratio, and total P of transgenic *Arabidopsis* plants overexpressing *ZmPHR1* and wild-type plants hydroponically supplied with high Pi and low Pi levels. Results are mean ±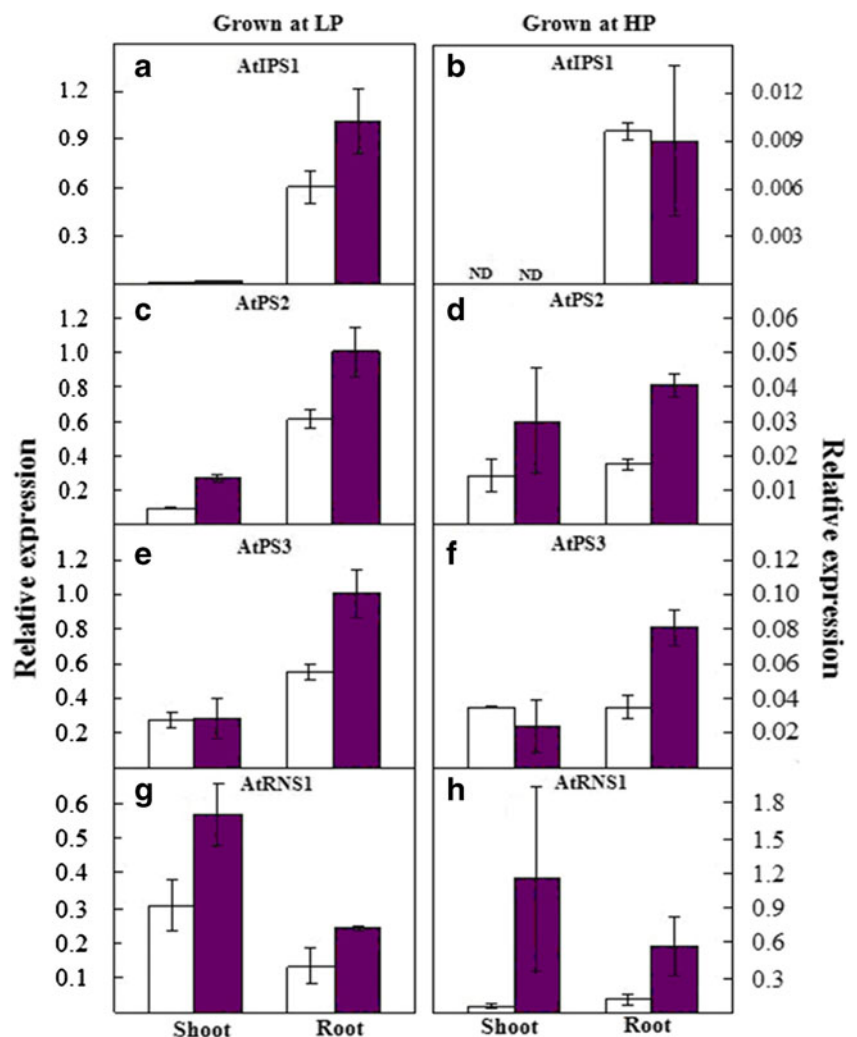 standard error (SE, *n*=3). Letters after the SE values of a particular parameter indicate whether the genotype had a significant influence within each P level ($P<0.05$), as determined by analysis of variance (one-way ANOVA) followed by least significance difference (LSD) post-hoc tests. *DW* Dry weight

P level	Genotype	Shoot DW (mg)	Root DW (mg)	Root to shoot ratio	Total P ($\mu mol\,g^{-1}$ DW)
LP (10 μM)	WT	57.5±1.2 c	11.9±1.8 b	0.206±0.002 a	145.0±12.3 b
	T	94.8±2.8 b	12.8±0.1 ab	0.135±0.005 b	164.8±4.3 b
HP (1 mM)	WT	132.5±8.6 a	14.6±1.8 ab	0.110±0.009 c	267.2±8.8 a
	T	145.5±1.7 a	14.9±0.3 a	0.103±0.001 c	269.8±2.5 a

ZmPHR1 Upregulates Pi Starvation-Inducible Genes

The expression patterns of Pi-starvation-inducible genes, including *AtIPS1*, *AtPS2*, *AtPS3*, and *AtRNS1*, were examined in *ZmPHR1*-overexpressing *Arabidopsis* plants (Fig. 8). The upstream promoter regions of these four genes contain one or more P1BS elements, i.e., *PHR1*-binding sites (Rubio et al. 2001). Under LP conditions, *ZmPHR1* regulated the expression of *AtIPS1*, *AtPS2*, *AtPS3* and *AtRNS1*, inducing various levels of expression. The expression of all four genes increased significantly in the roots of *ZmPHR1*-overexpressing plants compared to wild-type plants (Fig. 8a, c, e, g). The expression of the four genes was also upregulated by *ZmPHR1* in shoots; however, only the expression of *AtPS2* and *AtRNS1* obviously increased (Fig. 8c, g). Under HP conditions, *AtPS2*, *AtPS3* and *AtRNS1* were also upregulated in the roots of *ZmPHR1*-overexpressing plants (Fig. 8d, f, h). The expression of *AtRNS1* in *ZmPHR1*-transgenic plants was higher than that in the wild-type, irrespective of Pi treatment (Fig. 8g, h).

Overexpression of *ZmPHR1* Upregulates Several Pi Transporters

To investigate whether the Pi transport system is regulated by *ZmPHR1*, the expression levels of four high-affinity Pi transporters (Pht1;2, Pht1;4, Pht1;8 and Pht1;9) were analyzed by qPCR in wild-type and transgenic *Arabidopsis* plants (Fig. 9). A striking upregulation of Pht1;2, Pht1;8, Pht1;9, was observed (Fig. 9a, e, g) under LP conditions and Pht1;2 and Pht1;9 were also upregulated under HP conditions (Fig. 9b, h) in the roots of *ZmPHR1*-overexpressing plants compared to wild-type plants. The expression of these three genes was not detectable in the shoots of both genotypes, irrespective of Pi treatment (Fig. 9a, b, e, f, g, h). Moreover, the expression levels of Pht1;2, Pht1;8 and Pht1;9 in roots of *ZmPHR1*-overexpressing plants were higher under LP conditions than under HP conditions. This result suggested that *ZmPHR1* regulates the expression of Pht1;2, Pht1;8 and Pht1;9 in roots, particularly under LP conditions. There was no significant difference in expression

Fig. 8 Relative expression of *AtIPS1* (**a**, **b**), *AtPS2* (**c**, **d**), *AtPS3* (**e**, **f**) and *AtRNS1* (**g**, **h**) analyzed by qPCR in shoots and roots of wild-type *Arabidopsis* (*white columns*) and *ZmPHR1*-overexpressing transgenic lines (*purple columns*). Both genotypes grown in hydroponics at LP (10 μM Pi) and HP (1 mM Pi) conditions, respectively. *AtActin* was used as a reference gene. *ND* Not detectable. *Error bars* Standard error (*n* = 3). Note different scales was used for calibration analysis of different genes

Fig. 9 Relative expression of *Pht1;2* (**a, b**), *Pht1;4* (**c, d**), *Pht1;8* (**e, f**) and *Pht1;9* (**g, h**) analyzed by qPCR in shoots and roots of wild-type *Arabidopsis* (*white columns*) and *ZmPHR1*-overexpressing transgenic lines (*purple columns*). Both genotypes grown in hydroponics at LP (10 μM Pi) and HP (1 mM Pi) conditions, respectively. *AtActin* was used as a reference gene. *ND* Not detectable. *Error bars* Standard error (*n* = 3). Note different scales was used for calibration analysis of different genes
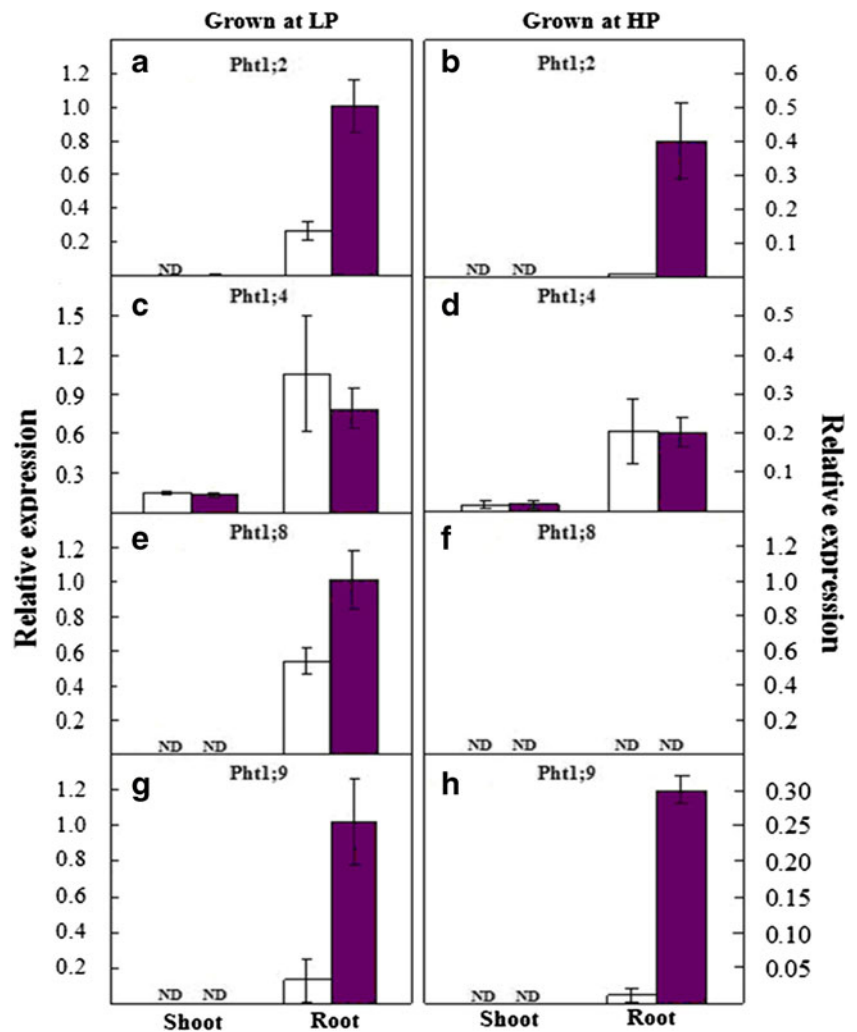
of Pht1;4 in the roots and shoots of both genotypes (Fig. 9c,d), implying that *ZmPHR1* might not regulate expression of Pht1;4.

Discussion

Many important traits are under complex polygenic control. The incorporation of a single gene encoding a qualitative trait is often insufficient when attempting to fully activate a target pathway by genetic manipulation. TFs that act as the main regulators of metabolic pathways are excellent candidates for modifying complex traits in crop plants, and TF-based technologies are likely to play a prominent role in the successful production of the next generation of bioengineered crops. PUE has been established as a quantitative characteristic under polygenic control, and plant biomass and Pi concentration are two key indexes for screening PUE genotypes. Our results indicate that a series of genes involved in the Pi-starvation metabolism were up-regulated in *Arabidopsis* plants overexpressing *ZmPHR1*, which

improved plant growth and increased Pi content in shoots under low-Pi conditions compared to wild-type plants. These results demonstrate the utility of introducing TFs into plants to alter plant metabolism, thus producing plants with superior characteristics.

A series of Pi-starvation response genes function in the Pi rescue system by solubilizing or releasing Pi from organic sources, which increases the capacity of plants to recycle internal Pi (Abel et al. 2002; Bari et al. 2006; Raghothama 1999). Under Pi-starvation conditions, RNases play an important role in the remobilization process (Bariola et al. 1994). *AtIPS1* inhibits the cleavage of PHO2 by miR399 and controls P homeostasis (Bari et al. 2006; Lin et al. 2008). *AtPS2* is a pyrophosphate-specific phosphatase, and *AtPS3* is a glycerol-3-phosphate permease. These genes are responsive to Pi starvation (Devaiah et al. 2009; May et al. 2011). P1BS elements, which are the target sequences of PHR1, exist in the upstream regions of the promoters of *AtIPS1*, *AtRNS1*, *AtPS2* and *AtPS3*. Loss of *AtPHR1* reduces the expression of *AtIPS1*, and the overexpression of PHR1 in WT and *phr1* mutant *Arabidopsis* plants leads to

the elevated expression of *AtIPS1* (Rubio et al. 2001; Nilsson et al. 2007). In rice, *OsIPS1* and *OsIPS2* are homologs of *AtIPS1* and have the same function as *AtIPS1*. In *OsPHR2*-overexpressing plants, expression of *OsIPS1* and *OsIPS2* increased in shoots and roots under Pi-starvation conditions (Hou et al. 2005; Zhou et al. 2008). In this study, *AtRNS1* expression in overexpressing *ZmPHR1*-transgenic *Arabidopsis* plants was higher than in wild type, irrespective of Pi regime. This was similar to the effect of *AtPHR1* on *AtRNS1* expression (Nilsson et al. 2007) and indicates that *AtRNS1* expression depends strongly on *ZmPHR1* levels. The expression of *AtIPS1*, *AtPS2* and *AtPS3* were enhanced greatly in roots under LP conditions. These results show that the overexpression of *ZmPHR1* greatly increases the expression of *AtIPS1*, *AtRNS1*, *AtPS2* and *AtPS3* in transgenic *Arabidopsis* plants compared to wild types under LP conditions, especially in roots.

High-affinity phosphate transporters play an important role under LP conditions (1–10 μM Pi) (Raghothama 2000). Members of the high-affinity phosphate transporter family Pht1 are located in the plasma membrane. These transporters acquire Pi from the rhizosphere, as most of them are expressed in root epidermal cells (Muchhal et al. 1996). Pht1;2 (*AtPT1*), Pht1;4 (*AtPT2*), Pht1;8 and Pht1;9 are members of the Pht1 family. Genes encoding these transporters contain P1BS elements in the upstream regions of their promoters and are regulated by *AtPHR1*. Nilsson et al. (2007) found that *AtPHR1* regulated only the expression of Pht1;8 and Pht1;9 in the shoots of plants under normal P conditions. The expression of *OsPT9*, which is related closely to Pht1;8 and Pht1;9, was increased substantially in the shoots and roots of *OsPHR2*-overexpressing plants under normal P conditions (Zhou et al. 2008). Pht1;4 has been reported to contribute to Pi uptake (Misson et al. 2004). In the current study, *ZmPHR1*, like *AtPHR1* (Nilsson et al. 2007), does not affect the expression of Pht1;4. Under LP conditions, the expression of Pht1;2, Pht1;8, and Pht1;9 in roots of *ZmPHR1*-overexpressing plants was notably higher than in wild type. Therefore, *ZmPHR1* participates in Pi transportation and utilization under Pi-starvation conditions. Together, these results suggest that overexpressing *ZmPHR1* upregulates the expression of Pht1;2, Pht1;8 and Pht1;9 in plant roots under LP conditions.

In summary, the overexpression of a single TF gene, *ZmPHR1*, had pleiotropic effects on numerous metabolic pathways in *Arabidopsis* plants, as overexpression of this gene stimulated Pi-starvation signaling and triggered the Pi-starvation response. Although it is currently unknown whether the expression of this TF has a direct or indirect effect on metabolism, our results highlight the possibility that enhancing *ZmPHR1* expression is a feasible strategy for improving Pi assimilation and use in crop plants, in particular maize under low-Pi conditions. Transgenic maize plants overexpressing *ZmPHR1* have been generated in our laboratory and will be the subject of future studies.

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